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Brief report
Human osteoarthritic chondrocytes are impaired in matrix metalloproteinase-13 inhibition by IFN-γ due to reduced IFN-γ receptor levels

R. Ahmad, M. El Mabrouk, J. Sylvester and M. Zafarullah*

Département de Médecine, Université de Montréal, Centre de recherche du CHUM (CRCHUM) Hôpital Notre-Dame, Montreal, Quebec, Canada H2L 4M1

Summary

Objective: Human osteoarthritic (OA) cartilage type-II collagen is preferentially cleaved by the proinflammatory cytokine-induced matrix metalloproteinases-13 (MMP-13). Interferon-gamma (IFN-γ) potently inhibits interleukin-1 (IL-1)-induced MMP-13 expression in healthy chondrocytes. Our goal was to study the previously unknown impact of IFN-γ on MMP-13 in OA and compare the levels and functional activity of IFN-γ receptor (IFN-γR1) in healthy and OA chondrocytes.

Methods: Chondrocytes were obtained from OA patients and non-arthritic control subjects and treated with IL-1 + or − IFN-γ. MMP-13 mRNA and protein expression were measured by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. IFN-γR1 expression was assessed by flow cytometry, immunoprecipitation and immunohistochemistry with fluorescein-labeled antibody. IFN-γR1 was neutralized with its antibody and signal transducer and activator of transcription 1 (STAT1) phosphorylation analyzed by Western blotting. OA chondrocytes were also transfected with control and IFN-γR1 expression vectors.

Results: OA chondrocytes displayed a drastically impaired MMP-13 suppression by IFN-γ compared to control cells. IFN-γR1 levels were significantly decreased in OA chondrocytes as assessed by flow cytometry, immunoprecipitation and immunohistochemistry. Consequently, IFN-γ-stimulated STAT1 phosphorylation mediated by IFN-γR1 was also considerably reduced in OA patient chondrocytes. IFN-γR1 overexpression in OA cells restored MMP-13 suppression by IFN-γ.

Conclusions: Ability of IFN-γ to suppress IL-1-induced MMP-13 expression is diminished in OA chondrocytes due to decreased IFN-γR1 levels, activity and impaired downstream signal transduction. Therefore, IFN-γR1 modulation and weakened endogenous IFN-γ response may be important mechanisms in OA pathogenesis and cartilage degradation.

Key words: Osteoarthritis, Interferon-gamma, Receptor, Signal transduction, Matrix metalloproteinase-13.

Introduction

Proinflammatory cytokines such as interleukin-1 (IL-1) are believed to be the principal stimuli for driving matrix metalloproteinase (MMP)-mediated cartilage degradation in patients with rheumatoid arthritis (RA) and osteoarthritis (OA)[1]. Due to its preferential and efficient cleavage of cartilage-specific type-II collagen, MMP-13 is thought to be the major enzyme responsible for cartilage collagen degradation[2]. Interferon-gamma (IFN-γ) is expressed by bacterial and viral antigen-activated T lymphocytes and natural killer cells. Contradictory proinflammatory and anti-inflammatory roles for IFN-γ have been reported, as deficiency of IFN-γ or IFN-γ receptor (IFN-γR1) reduces[3] or enhances[4] susceptibility to collagen-induced arthritis. Despite lack of any major effect by recombinant IFN-γ in a clinical trial on RA patients[5], there is a renewed interest in anti-inflammatory, chondroprotective and antiosteoclastogenic effects of IFN-γ[6]. We have shown that IFN-γ potently inhibits IL-1-induced MMP-13 promoter activity and gene expression in chondrocytes from healthy subjects[7]. To investigate the status of OA cartilage response to IFN-γ, the current study compared previously unknown IFN-γ suppression response of MMP-13 and levels and activity of IFN-γR1 in control and OA chondrocytes.

*Address correspondence and reprint requests to: Dr M. Zafarullah, K-5255 Mailloux, Hôpital Notre-Dame du CHUM, 1560 Sherbrooke E, Montréal, Québec, Canada H2L 4M1. Tel: 1-514-890-8000-25690; Fax: 1-514-412-7612; E-mail: muhammad.zafarullah@umontreal.ca

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Methods

CHONDROCYTES AND RT-PCR

Chondrocytes were released enzymatically from the undamaged cartilage of the non-weight bearing region of femoral head of four patients (two males, two females; mean age, 60 years) with hip OA as reported before[8], who underwent joint replacement surgery at the Notre-Dame Hospital of CHUM. Institutional ethical committee approved utilization of these tissues. Control knee chondrocytes from three different subjects (two males, one female; mean age, 47.3 years) were obtained from Cambrex (Walkerville, MD). Both groups of chondrocytes at passage 2 showed their typical morphology and expressed cartilage-specific collagen II marker as shown below. MMP-13 and glyceraldehye-3-phosphate dehydrogenase (GAPDH) mRNA levels were measured by reverse transcription-polymerase chain reaction (RT-PCR) with MMP-13 specific primers[8] yielding 491 and 226 bp cDNA bands.

FLOW CYTOMETRY AND IMMUNOHISTOCHEMISTRY

OA and control chondrocytes were removed from the culture plates by incubating with 100 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS) on ice for 30 min, washed with cold PBS plus 0.2% bovine serum albumin (BSA) twice and incubated with Phycoerythrin (PE)-conjugated anti-IFN-γR1 mAb (BD Biosciences, Mississauga ON) or PE-conjugated mouse immunoglobulin G1 (IgG1) mAb, isotype control for 30 min on ice. After staining, cells were washed twice with PBS and fixed with cold freshly prepared 2% formaldehyde for 30 min followed by double washing with PBS prior to analysis on a Fluorescence-Activated Cell Sorting (FACS) Calibur Flow Cytomer (BD Biosciences). Control and OA chondrocytes grown in flat-bottomed 96-well plates were also stained with IFN-γR1 antibody followed by incubation with Fluorescein isothiocyanate (FITC)-goat anti-mouse secondary antibody.

WESTERN BLOTTING

For Western blotting, 3×10^6 cells were harvested and incubated for 30 min with lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 30 μl/ml Protease Inhibitor Cocktail Set III-Calbiochem and 1 mM Na_3VO_4) and separated by SDS-PAGE. Antibodies against Phospho-STAT1-Tyr-701, total STAT1 (Cell signaling Technology, Beverley, MA), Collagen II (Chemicon International, Temecula, CA) and beta-actin, (Sigma, Saint Louis, MO) were used at recommended dilutions. The blots were then washed and incubated for 2 h with Horse Radish Peroxidase-conjugated secondary antibody. Immunoreactive bands were developed using an Enhanced Chemiluminescent substrate (Amersham) and visualized by autoradiography. For MMP-13, supernatants were collected from the 3×10^6 cells cultured in the 9.5 cm^2 6-well plates (Costar 3516) and proteins precipitated with 10% trichloroacetic acid by keeping at −20°C for 30 min and then centrifuged at 14,000 rpm for 20 min at 4°C. The precipitated proteins were dissolved in 0.1 M NaOH. Sample loading buffer (100 mM Tris pH 6.8, 200 mM dithiothreitol, 4% SDS, 20% Glycerol, 0.02% Bromophenol blue) was added, boiled for 5 min and run on SDS-PAGE for separation of proteins. Antibody against MMP-13 (Sigma) was used at 1:500 dilution.

IMMUNOPRECIPITATION AND TRANSFECTION

Immunoprecipitation was done as described earlier[7]. Briefly, 3×10^6 cells were lysed on ice for 30 min and lysates centrifuged at 14,000 rpm for 10 min and supernatants were collected. One milligram of total protein was mixed with 1 μg/ml monoclonal anti-IFN-γR1 Ab (Chemicon International Inc) or mouse IgG at 4°C for overnight and then protein-A agarose beads were added and incubated for 3 h at 4°C. The precipitates bound to the beads were washed 5× with 1× RIPA buffer and resuspended in 2× SDS loading buffer and then boiled at 95°C for 5 min. Samples were centrifuged and supernatants analyzed by Western blotting. Receptor neutralization and transient transfections of Cytomegalovirus (CMV) promoter-based pCMV-XL5 and CMV-IFN-γR1 (Origene SC119876) expression vectors were performed as described earlier[7].

QUANTIFICATION

The bands were scanned and quantified by National Institute of Health Image software by dividing the values of test band by control band (beta-actin, GAPDH) (arbitrary units) followed by graphic presentation of the mean values. Differences between mean values of OA patients and controls for various parameters were tested for statistical significance using Student's *t* test. *P* values of <0.05 were considered significant.

Results

IFN-γ severely suppressed IL-1-induced MMP-13 mRNA without affecting the constitutive GAPDH mRNA expression in chondrocytes from control subjects [Fig. 1(A)]. IFN-γ alone did not affect basal MMP-13 expression [Fig. 1(E)]. Although OA condrocytes appeared to have slightly higher extent of MMP-13 mRNA induction by IL-1, it was similar after normalization with GAPDH control [Fig. 1(C)]. IFN-γ failed to completely suppress MMP-13 in OA cells [Fig. 1(A)]. MMP-13 protein levels analyzed by Western blotting were comparable (though slightly higher in OA) to those of mRNA [Fig. 1(B)]. Quantification of the bands demonstrated differential MMP-13 suppression responses of control and OA chondrocytes to IFN-γ [Fig. 1(C) and (D)]. This response was not due to insufficient IL-1 as dose−response showed maximal MMP-13 mRNA induction with the dose of 10 ng/ml used [Fig. 1(F)]. Furthermore, the dose of 300 units/ml IFN-γ was sufficiently high to effectively suppress MMP-13 mRNA and protein expression [Fig. 1(G)].

To investigate the mechanism of differential IFN-γ response of human OA chondrocytes, we tested the hypothesis that expression of the IFN-γR1 or its activity may be impaired in OA chondrocytes. IFN-γ receptor levels were measured on chondrocytes from three controls and four OA patients by flow cytometry. Jurkat cells expressing this receptor served as positive controls and yielded 92% positive cells (maximum). Human normal chondrocytes displayed higher percentage of (67−69% positive) IFN-γR1 staining [Fig. 2(A) left panels and (B)] compared to severely reduced percentage (30−34% positive) in OA patient chondrocytes [Fig. 2(A), right panels and (B)]. To further examine the changes in IFN-γR1 expression, immunoprecipitates from OA patient and control chondrocytes were subjected to Western blotting. Consistent with the positive control, Jurkat and peripheral blood mononuclear cells (PBMCs), a single band of about 90 kDa was reproducibly observed in all specimens. Diminished expression of IFN-γR1 protein band in OA chondrocytes was clearly observed by this alternative analysis. Based on the ratio of IFN-γR1: β-actin protein bands, the levels of IFN-γR1 protein expression were significantly different in the two groups [Fig. 2(C) and (D)]. The studied chondrocytes maintained their phenotype by expressing consistent levels of chondrocyte-specific type-II collagen marker as measured by Western blotting including purified type-II collagen as positive control [Fig. 2(E)]. Thus, poor response of OA chondrocytes to IFN-γ may be due to diminished expression of IFN-γR1. Indeed, immunohistochemistry with IFN-γR1 antibody on two control and two OA chondrocyte lines indicated decreased IFN-γR1 levels in OA cells [Fig. 2(F)].

STAT1 is a pivotal mediator of IFN-γ inhibition of MMP-13[7]. Further, IFN-γ signal transduction takes place primarily through JAK/STAT pathway[9]. To further study the mechanism of diminished IFN-γ response in OA chondrocytes and to test whether IFN-γ-induced STAT1 phosphorylation is impaired in OA patients, control and OA chondrocytes were treated with IFN-γ and lysates analyzed by immunoblotting with anti-STAT1 antibodies. The total STAT1 levels remained constant. However, IFN-γ-treated chondrocytes from OA patients displayed significantly reduced levels of tyrosine-phosphorylated STAT1 (48.50%) [Fig. 3(A) and (B)] compared to the elevated phosphorylation in control cells. To investigate whether the decreased pSTAT1 levels are receptor mediated, we performed receptor neutralization experiments and showed that IFN-γR1 neutralizing antibody and not IgG completely inhibited IFN-γ-stimulated STAT1 phosphorylation [Fig. 3(C)]. IFN-γR1 antibody alone had no effect [Fig. 3(D)]. We have previously shown that inhibition of MMP-13 by IFN-γ is also mediated by IFN-γR1[7].

To further explore the role of IFN-γR1 deficiency in hyporesponsiveness of OA chondrocytes, two lines of OA chondrocytes were transfected with the pCMV-XL5 or CMV-IFN-γR1 expression vector and then treated with IL-1 and IFN-γ. pCMV-XL5 vector had no effect on IL-1-induced MMP-13 suppression by IFN-γ. CMV-IFN-γR1 overexpression completely (patient 1) or partially (patient 2) restored suppression by IFN-γ (Fig. 4).

Discussion

We have demonstrated here for the first time that compared to controls, human OA chondrocytes have

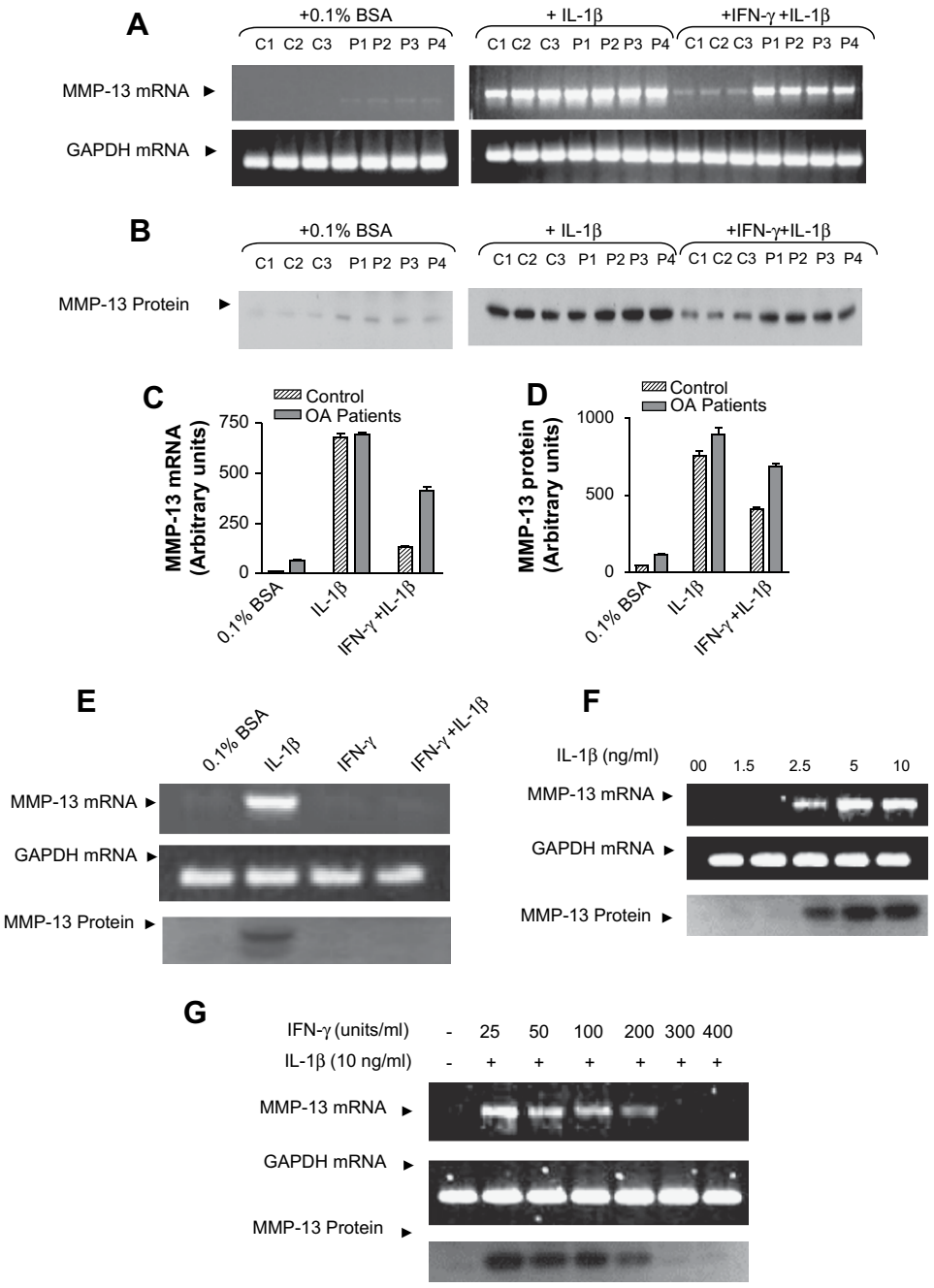

Fig. 1. Impaired suppression of IL-1-induced MMP-13 gene expression by IFN-γ in human OA chondrocytes. Human chondrocytes from different healthy control subjects (C) or OA patients (P) were treated with vehicle (0.1% BSA), IL-1β (10 ng/ml), IFN-γ (300 units/ml) alone or in combination for 24 h. Cells were harvested for MMP-13 (A, upper panel) and GAPDH (A, lower panel) mRNA analysis by RT-PCR. (B) The supernatants were used for MMP-13 protein (48 kDa, active enzyme) analysis by Western blotting. (C) Band densities of MMP-13 RT-PCR products were quantified in arbitrary units and normalized with GAPDH. The values are the mean and SE. (D) The densities of MMP-13 protein bands were quantified and shown as mean values with SE. The mean values of two groups differed significantly ($P < 0.05$). (E) Human chondrocytes were treated with vehicle control (BSA), IL-1, IFN-γ or both together for 24 h and MMP-13 mRNA and protein analyzed as above. IFN-γ alone does not affect MMP-13 expression. (F) Dose-dependent induction of MMP-13 mRNA and protein by IL-1β providing justification for the use of 10 ng/ml dose. (G) Dose-dependent inhibition of IL-1-induced MMP-13 mRNA and protein by IFN-γ in human OA chondrocytes justifying the 300 Units/ml as the effective dose.

significantly reduced repression of MMP-13 induction partly due to diminished IFN-γR1 levels and resulting impaired signal transduction through STAT1. We have further shown directly the expression of IFN-γR1 on chondrocytes.

The elevated basal expression of MMP-13 mRNA and protein in OA patients is in agreement with the previous results which also reconfirm the disease-activated state of OA chondrocytes and their hypersensitivity to IL-1[8]. Reproducibly

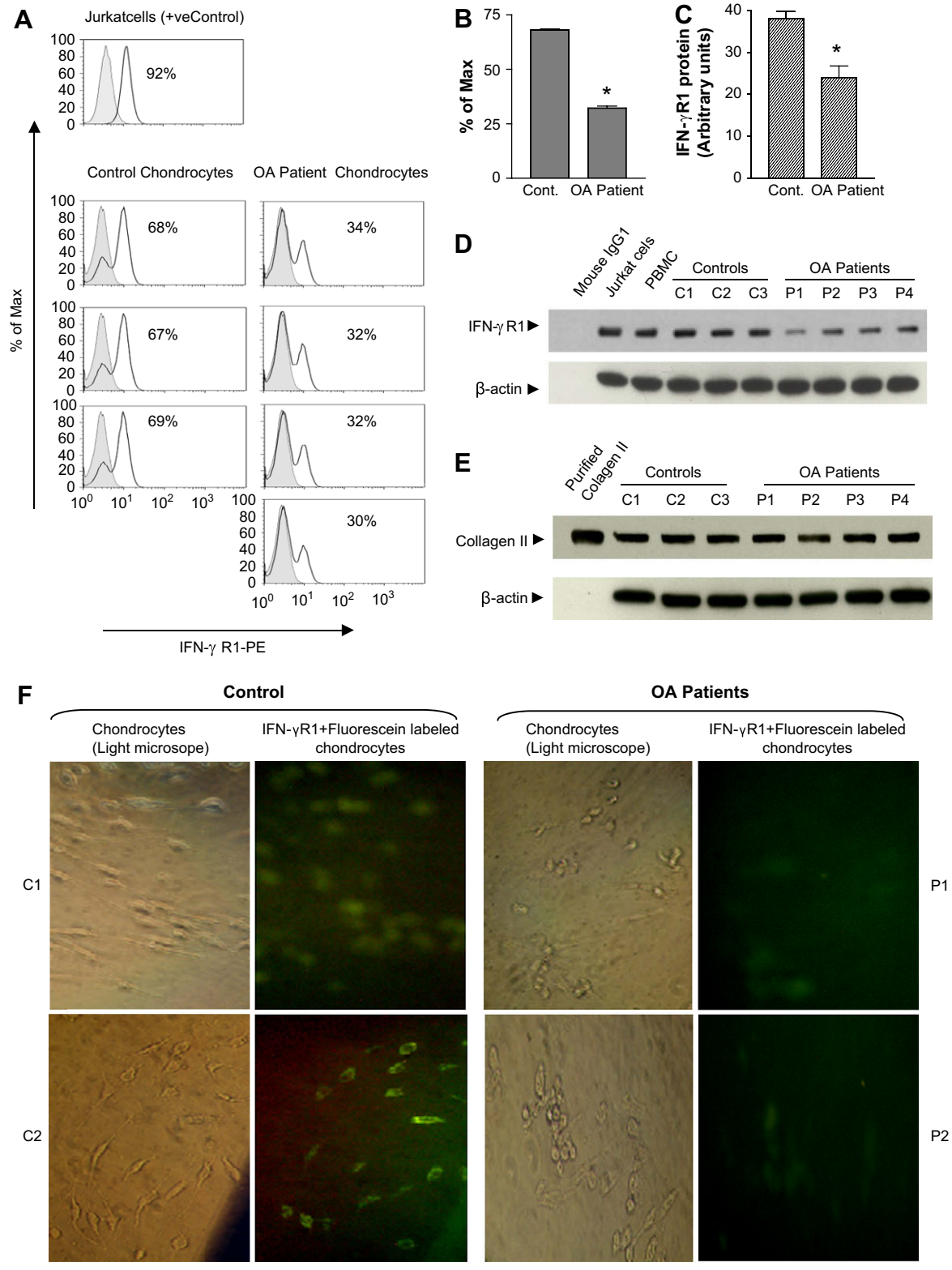

Fig. 2. Analysis of IFN-γR1 expression on the chondrocytes derived from patients with OA (P) and control (C) subjects. (A) Chondrocyte surface expression of IFN-γR1 was evaluated by flow cytometry using Jurkat cells (upper panel) as positive controls. The histograms represent the percentage of cells expressing IFN-γR1 (right panels, white histogram or background of mouse IgG1 negative control gray histogram). The histogram is representative of three independent experiments for each patient. (B) The mean percentages of chondrocytes expressing IFN-γR1. A star shows statistically significant ($P < 0.05$) difference between the two groups. (C) The band densities of OA patient and control IFN-γR1 protein were presented as mean values and SE, which differed significantly. (D) Total cell lysates were immunoprecipitated with anti-IFN-γR1 antibody or control non-immune mouse IgG, resolved by SDS-PAGE and analyzed by Western immunoblotting with

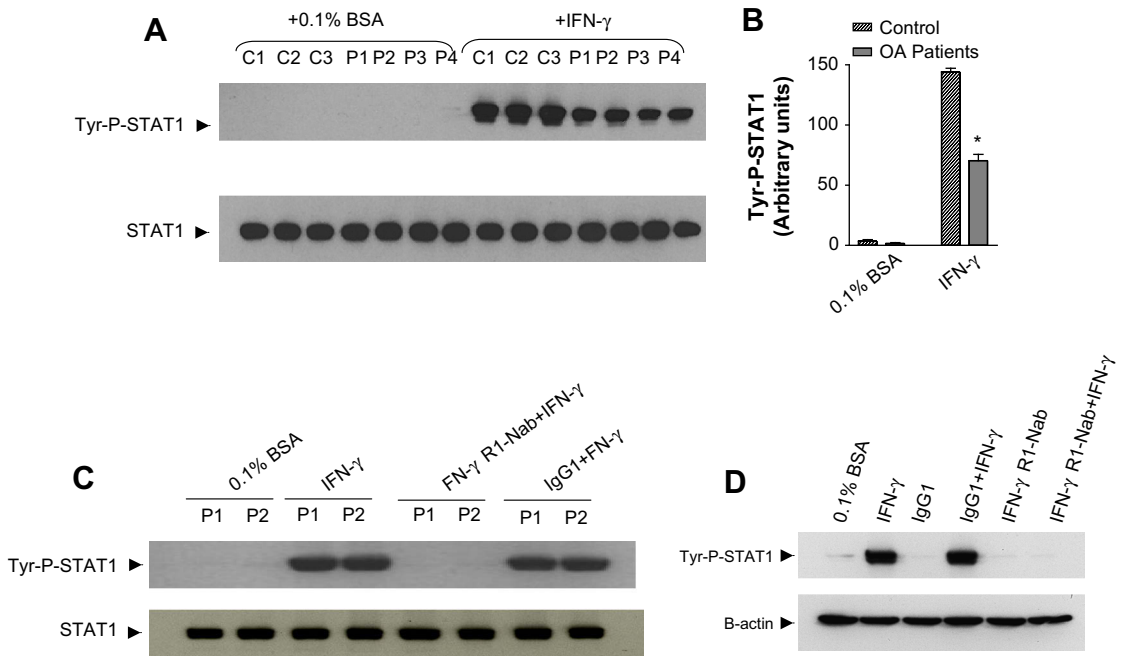

Fig. 3. Differential IFN-γ-stimulated STAT1 phosphorylation in control and OA chondrocytes. Chondrocytes were incubated for 30 min with IFN-γ or 0.1% BSA and equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotting with anti-phospho-STAT1 (A) and respective total (lower panels) antibodies. (B) The normalized densities of STAT1 bands shown in the form of mean values with SE. (C) Neutralization of IFN-γR1 blocks IFN-γ-stimulated STAT1 phosphorylation. Human OA chondrocytes from two different individuals were grown to confluence and treated with control IgG or neutralizing IFN-γR1 antibody (Nab) for 1 h and then stimulated with IFN-γ for 20 min followed by Western blotting with anti-phospho- and total STAT1. The resulting bands are shown. (D) IFN-γR1 Nab alone did not affect STAT1 phosphorylation.

diminished extent of MMP-13 suppression by IFN-γ in OA patient vs control chondrocytes indicates an inherent defect in the protective IFN-γ response of OA chondrocytes possibly due to arthritis. A prevailing proinflammatory environment in the joints may be conducive to the observed poor IFN-γ responsiveness. The reported increased susceptibility of IFN-γ- or IFN-γR1-deficient mice to collagen-induced arthritis (a RA-like model) and cartilage and bone loss[10] may also be partly due to defective suppression of MMPs.

By receptor blockade experiments, we have indirectly demonstrated the requirement of IFN-γR1 for repression of MMP-13 by IFN-γ[7]. However, IFN-γ receptor has not been detected directly in human or animal chondrocytes. Here we show for the first time that both control and OA chondrocytes express IFN-γR1. Because the reasons for hypo-effectiveness of IFN-γ in suppressing MMP-13 expression in OA chondrocytes were unknown, we investigated the hypothesized role of IFN-γR1 by measuring its levels in both groups of chondrocytes. Most interestingly, a consistently diminished expression of IFN-γR1 in OA chondrocytes from several patients was demonstrated by multiple approaches of flow cytometry, immunoprecipitation/Western blot analysis and immunohistochemistry. Additionally, constant type-II collagen expression demonstrated that chondrocyte phenotype was stable and that the receptor reduction was selective and was not due to overall changes in chondrocyte metabolism as confirmed further by β-actin levels. Previously, IFN-γR1 expression was found more abundant in rheumatoid (11/11 patients) than OA (3/8 patients) synovial tissue[11]. Depression of IFN-γR1 could be a general feature of human OA tissues that should be studied further. It is noteworthy that in animal models, IFN-γR1 deficiency leads to RA-like symptoms in multiple joints or an increased susceptibility to arthritis[4,12].

Control and OA chondrocytes displayed significantly differential IFN-γ signal transduction response as analyzed by STAT1 phosphorylation. The reduced STAT1 tyrosine phosphorylation in OA chondrocytes strongly correlates with the aforementioned impaired receptor levels in patient cells and is indeed mediated by the receptor as shown by the receptor neutralization experiments. Thus IFN-γR1 levels and functionality are important in generation of IFN-γ responses in chondrocytes. Previous reports did not study STAT1 levels in cartilage but have shown enhanced STAT1 levels in active RA relative to OA synovial tissue[13,14]. This may also be true for cartilage, which is a distinct and essential tissue for weight bearing, joint flexibility and mobility. The lower IFN-γ response

anti-IFN-γR1 antibody (upper panel). Jurkat cells and PBMCs extracts were used as positive controls. Lysates (20 μg) were probed for beta-actin analysis (lower panel). (E) For monitoring chondrocyte metabolism and phenotype, collagen II (upper panel) and beta-actin (lower panel) protein levels in both groups of chondrocytes were determined by Western blotting using purified type-II collagen as positive control (lane 1). (F) Detection of IFN-γR1 in chondrocytes by using fluorescein-labeled secondary antibody. Cells were grown in 96-well flat bottom microplates for 48 h. Cells were washed with PBS containing 0.2% BSA and incubated with a mouse anti-human IFN-γR1 monoclonal antibody (2 μg/ml; Chemicon International Cat# MAB1154) for 45 min. Cells were washed twice and then probed with FITC-goat anti-mouse secondary antibody (BD Biosciences Cat# 58859) (2 μg/ml). Cells washed and then fluorescent images were recorded through fluorescence microscopy. C = Control; P = OA patients. Light microscope images of the corresponding regions are also shown.

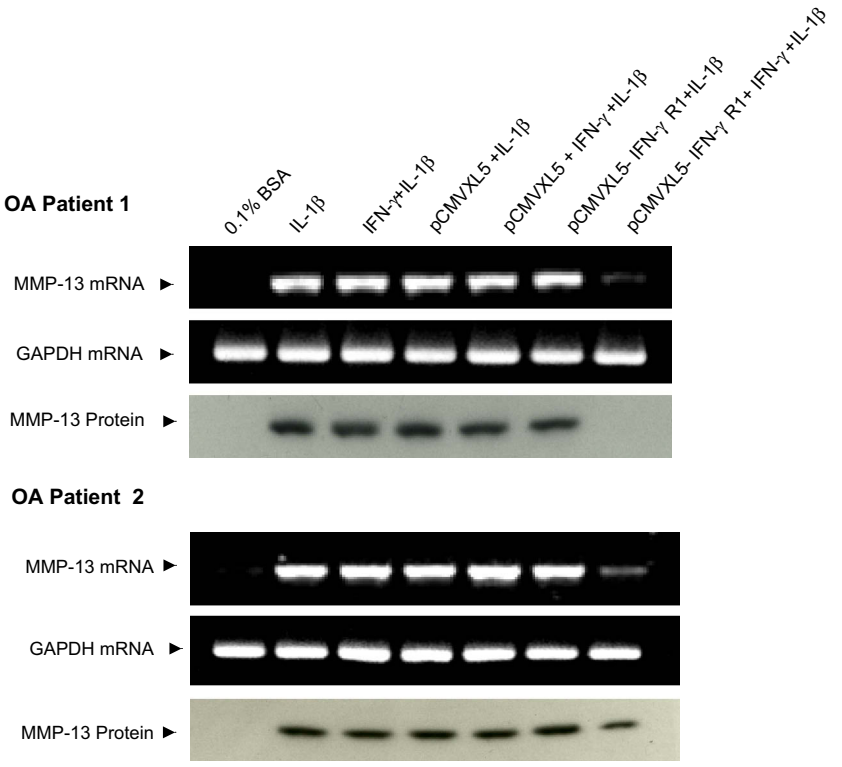

Fig. 4. Transfection of IFN-γR1 vector restores the ability of IFN-γ to repress IL-1-induced MMP-13 expression in OA chondrocytes. Cells from two different OA patients were transfected with 1 μg of pCMV-XL5 (empty vector) or CMV-IFN-γR1 expression vector and then treated with IL-1 and IFN-γ for 24 h as indicated. Results of MMP-13 and GAPDH control mRNA RT-PCR as well as MMP-13 protein Western blot are depicted.

of STAT1 phosphorylation in OA cartilage-derived chondrocytes further supports an intrinsic defect. Interferons limit IL-1-stimulated inflammation and tissue destruction by inhibiting inflammatory mediators (cytokines, Cox-2) and MMPs (MMP-3, MMP-9)[15]. Such impairment of the defensive mechanism at multiple levels could lead to enhanced cartilage degradation. Whether this deficiency is a cause or consequence of OA remains to be studied further.

In summary, OA patients exhibit a clear tendency of impaired IFN-γ response and MMP-13 suppression due to diminished IFN-γR1 levels and subsequent signal transduction through STAT1. Considering the reported protective roles of IFN-γ in limiting inflammation and cartilage destruction, such an impaired IFN-γ response in patients may be a pivotal contributing factor for cartilage degeneration in arthritis and could constitute a therapeutic target.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

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